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Enhanced activation of axonally transported stress-activated protein kinases in peripheral nerve in diabetic neuropathy is prevented by neurotrophin-3

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Summary

The objective was to determine whether stress-activated protein kinases (SAPKs) mediated the transfer of diabetes-induced stress signals from the periphery to somata of sensory neurons. Thus, we characterized axonal transport of SAPKs in peripheral nerve, studied any alteration in streptozotocin (STZ)-diabetic rats and examined effects of neurotrophin-3 (NT-3) on diabetes-induced events. We demonstrate that c-jun N-terminal kinase (JNK) and p38 are bidirectionally axonally transported at fast rates in sciatic nerve. In STZ-diabetic rats the relative levels of retrograde axonal transport of phosphorylated (activated) JNK and p38 were raised compared with age-matched controls (all data are in arbitrary units and expressed as fold increase over control: JNK 54–56 kDa isoforms, control 1.0 ± 0.19, diabetic 2.5 ± 0.26; p38, control 1.0 ± 0.09, diabetic 2.9 ± 0.52; both $P < 0.05$). Transport of total enzyme levels of JNK and p38 and phosphorylated extracellular signal-regulated kinase (ERK) was not significantly altered and anterograde axonal transport of phosphorylated JNK and p38 was unaffected by diabetes. The transcription factor ATF-2, which is phosphorylated and activated by JNK and p38, also exhibited elevated retrograde axonal transport in STZ-diabetic animals (control 1.0 ± 0.07, diabetic 3.0 ± 0.41; $P < 0.05$). Treatment of STZ-diabetic animals with 5 mg/kg human recombinant NT-3 prevented activation of JNK and p38 in sciatic nerve (phosphorylated JNK, control 1.0 ± 0.09, diabetic 1.95 ± 0.35, diabetic + NT-3 1.09 ± 0.12; $P < 0.05$ diabetic versus others; phosphorylated p38, control 1.0 ± 0.16, diabetic 4.7 ± 0.9, diabetic + NT-3 1.19 ± 0.18; $P < 0.05$ diabetic versus others). The results show that JNK and p38 are transported axonally and may mediate the transfer of diabetes-related stress signals, possibly triggered by loss of neurotrophic support, from the periphery to the neuronal soma.

Keywords: DRG; neurotrophic factors; NT-3; peripheral neuropathy; SAPKs

Abbreviations: CGRP = calcitonin gene-related peptide; DRG = dorsal root ganglia; ERK = extracellular signal-regulated kinase; JIP-1 = JNK inhibitory protein 1; JNK = c-jun N-terminal kinase; NGF = nerve growth factor; NT-3 = neurotrophin-3; SAPK = stress-activated protein kinase; STZ = streptozotocin

Introduction

Diabetic sensory neuropathy in humans is associated with a spectrum of structural changes in peripheral nerve that includes axonal degeneration, paranodal demyelination and loss of myelinated fibres, the latter probably the result of dying back of distal axons (Thomas and Tomlinson, 1993; Yagihashi, 1997). In the streptozotocin (STZ)-diabetic rat and Bio-Breeding rat animal models of type I diabetes, similar structural abnormalities in peripheral nerve have been observed (Yagihashi, 1997). The Diabetes Control and Complications Trial concluded that control of hyperglycaemia is still the ideal means of preventing the appearance of complications in diabetes, such as peripheral neuropathy (Diabetes Control and Complications Trial Research Group, 1993). However, such a goal remains an unrealized ideal and research continues to focus on biochemical transducers downstream from hyperglycaemia that may directly induce neuropathic sensory nerve damage.

The stress-activated protein kinase (SAPK) subgroup of mitogen-activated protein kinases are activated by a range of cellular stresses. Among the most commonly studied of these

are hyperglycaemia, hypertonicity, treatment with cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor α (TNF-α), oxidative stress and ultraviolet irradiation (Whitmarsh and Davis, 1996; Davis, 2000; Weston and Davis, 2002). In PC12 cells, SH-SY5Y cells, embryonic cortical neurons and embryonic peripheral neurons, the members of the SAPK family are activated by glutamate (Ackerley *et al.*, 2000; Brownlees *et al.*, 2000), hyperglycaemia (Cheng and Feldman, 1998), hyperosmolarity (Giasson and Mushynski, 1997) and changes in neurotrophic support (Xia *et al.*, 1995; Deshmukh and Johnson, 1997). The SAPK family includes extracellular signal-regulated kinase 1/2 (ERK 1/2), c-jun N-terminal kinase (JNK; comprising three genes, termed JNK1, 2 and 3) and p38/HOG (Whitmarsh and Davis, 1996; Davis, 2000; Harper and LoGrasso, 2001; Weston and Davis, 2002). Upon activation, this family of kinases bind to the transcription factors c-jun (JNK only), ATF2 and ELK-1 and phosphorylate the activation domains favouring DNA binding (Whitmarsh and Davis, 1996; Davis, 2000; Weston and Davis, 2002). Events downstream of transcription factor activation are not well defined in neurons, but in PC12 cells and sympathetic neurons the activation of JNK and p38, triggered by removal of nerve growth factor (NGF), induces apoptosis (Xia *et al.*, 1995; Deshmukh and Johnson, 1997).

JNK1 and JNK2 combine to regulate apoptosis during development of the mouse brain (Kuan *et al.*, 1999). In the adult CNS there is heterogeneous expression of JNK and activation in response to extracellular stress (Carletti *et al.*, 1995; Xu *et al.*, 1997); for example, kainate-induced excitotoxicity in the hippocampus is mediated via activation of JNK3 (Yang *et al.*, 1997). In the PNS, axotomy of sensory neurons induces rapid and long-term activation of JNK within the lumbar dorsal root ganglia (DRG) (Kenney and Kocsis, 1998). The mechanism of JNK activation is unknown but proinflammatory cytokines, including IL-1β and TNF-α, are expressed in nerve trunks after axotomy and these may activate neuronal SAPKs (Rotshenker *et al.*, 1992; Murphy *et al.*, 1995). Following axotomy, neurons are also exposed to a relative loss of neurotrophic support (Richardson, 1991).

Our previous studies in STZ-diabetic and Bio-Breeding rats show that JNK, p38 and ERK are activated in lumbar DRG and sural nerve and could mediate neurodegeneration (Fernyhough *et al.*, 1999). We have proposed that activation of JNK and ERK may mediate aberrant neurofilament phosphorylation in sensory neurons in diabetes and induce loss of axon calibre and eventual dying back of nerve endings (Fernyhough *et al.*, 1999). Furthermore, activation of p38 and ERK in cultured sensory neurons was shown to be associated with oxidative stress, and protection from cell death could be afforded by inhibition of this activation (Purves *et al.*, 2001).

The transport of signalling molecules downstream from tyrosine kinase receptors has been demonstrated previously in peripheral nerve. For example, ERK and the transcription factor ATF2 are transported bidirectionally in sciatic nerve (Delcroix *et al.*, 1999; Averill *et al.*, 2001; Reynolds *et al.*, 2001). The SAPKs have been localized to the axons of peripheral nerve (Fernyhough *et al.*, 1999) and, therefore, the present study was designed to test whether SAPKs were transported in axons of the sciatic nerve of adult rats. Additionally, the aim was to determine whether the activation state of transported SAPKs was affected by type I diabetes, and if so, to examine effects of treatment with neurotrophin-3 (NT-3), which is known to offer protection against experimental diabetic neuropathy (Mizisin *et al.*, 1999).

Methods

Induction of diabetes

Male Wistar rats were made diabetic for 8 weeks (or for 14 weeks in the NT-3 study; see below) by a single intraperitoneal injection of STZ (55 mg/kg; Sigma, St Louis, MO, USA). The level of glucose in tail vein blood was measured using a reflectance meter operated by a glucose oxidase strip (Reflolux II BCL; Boehringer Mannheim), and it was >27 mmol/l for all STZ-injected rats. At death, plasma glucose levels for all groups of diabetic rats were >20 mmol/l (plasma glucose levels for control rats ranged from 10 to 11 mmol/l). In the axonal transport study, the body weights for age-matched control rats ranged between 450 and 500 g, while all groups of diabetic rats weighed 300–350 g at the end of the study. All animal procedures followed the strict guidelines of the UK Home Office Regulations and were in accordance with the UK Animals (Scientific Procedures) Act 1986.

NT-3 treatment study

Two groups of Wistar rats were made diabetic for 14 weeks. One group of diabetic rats received subcutaneous injections of 5 mg/kg human recombinant NT-3 (a gift from Regeneron Pharmaceuticals, Tarrytown, NY, USA) three times weekly for the final 10 weeks of the 14-week diabetes study. Body weights for age-matched control rats ranged from 530 to 710 g, while all groups of diabetic rats weighed 290–445 g at study end. For all diabetic animals, the motor nerve conductance velocity and sensory nerve conductance velocity (as measured by the H-reflex) were significantly reduced. The efficacy of NT-3 treatment was confirmed by reversal of the sensory nerve conductance velocity deficit, as described previously (Tomlinson *et al.*, 1996; Mizisin *et al.*, 1998, 1999) (data not shown). Animals were killed by a blow to the head and exsanguination and 1-cm segments of sciatic nerve were isolated and frozen immediately on dry ice in preparation for quantitative western blotting.

Quantification of axonal transport using western blotting

Animals were anaesthetized using halothane, the sciatic nerve was exposed unilaterally and double ligatures 1 cm apart were placed at mid-thigh. This double ligature procedure for the

measurement of axonal transport has been described by Raivich *et al.* (1991). Briefly, the double ligatures were applied for 6 or 12 h, the rats were killed, and 0.5 cm of sciatic nerve proximal (for anterograde accumulation), distal (for retrograde accumulation) and intermediate to each ligature was extracted and frozen in liquid nitrogen. For calculation of retrograde axonal transport, the western blot signal for the intermediate nerve segment was subtracted from the signal for the distal or retrograde accumulation. The intermediate value was derived mainly from local synthesis between the ligatures (and induced by the ligature), whereas the distal (retrograde) accumulation included local synthesis and the accumulated material. These samples and the sciatic nerve segments from the NT-3 study were homogenized using a Polytron (Kinematica, Lucerne, Switzerland) in 0.1 mM PIPES (pH 6.9), 5.0 mM $MgCl_2$, 5.0 mM EGTA, 0.5% Triton X-100, 20% glycerol, 1.0 mM phenylmethylsulphonyl fluoride and a mixture of protease inhibitors (1.0 μg/ml pepstatin A, 1.0 μg/ml leupeptin, 10 μg/ml benzoyl-L-arginine methyl ester, 10 μg/ml *p*-tosyl-L-arginine methyl ester, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml L-1-tosylamide-2 phenylethylchloromethyl ketone and 7 μg/ml aprotinin). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (10% acrylamide) was performed on 10 μg of protein, and the separated proteins were transferred to nitrocellulose [Amersham ECL (enhanced chemiluminescence) membrane; Amersham Biosciences, Little Chalfont, UK] using a graphite blotter. Primary antibodies used were a polyclonal to total JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA, code FL; diluted 1 : 1000) and a monoclonal antibody to phosphorylated JNK (Santa Cruz, code G-7; 1 : 200; phosphorylated on Thr-183 and Tyr-185); antibodies to total p38 (New England Biolabs, Beverley, MA, USA, code 9212; 1 : 1000) and phosphorylated p38 (Biosource International, Camarillo, CA, USA, code 44–684; 1 : 2000; phosphorylated on Thr-180 and Tyr-182); polyclonal antibodies to total ATF2 (New England Biolabs, code 9222; 1 : 1000) and phosphorylated ATF2 (New England Biolabs, code 9225; 1 : 1000; phosphorylated on Thr-69/71); and polyclonal antibody to phosphorylated ERK (New England Biolabs, code 9101; 1 : 4000; phosphorylated on Thr-202 and Tyr-204). Detection was achieved using the New England Biolabs phototope-HRP system. The relative levels of protein were determined using an image analyser (AI, Cambridge, UK). Following ECL detection, all blots were stained with Indian ink to confirm even loading of protein.

Immunohistochemistry for JNK and p38 in sciatic nerve

Three normal rats and three rats that had undergone unilateral sciatic nerve ligature for 12 h (as described above) were used for immunohistochemical analysis of axonal transport and SAPK localization to axons using standard techniques (Priestley, 1997). The animals were anaesthetized with sodium pentobarbital (60 mg/kg) and perfused through the ascending aorta with saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The mid-sciatic nerve (covering ligatured area) and intact nerve from unoperated animals were removed, postfixed for 2 h in the same fixative and cryoprotected in 15% sucrose overnight. Tissues were frozen and sectioned at 8 μm either longitudinally (for SAPK accumulation at crush site) or transversely (for localization of SAPKs to axon). Sections were stained using indirect immunofluorescence histochemistry with polyclonal antibodies directed against total JNK (Santa Cruz Biotechnology, code FL; diluted 1 : 100); phosphorylated JNK (Promega, Madison, WI, USA, code v1211; 1 : 200; phosphorylated on Thr-183 and Tyr-185); antibodies to total p38 (Santa Cruz Biotechnology, code FL; diluted 1 : 100) and phosphorylated p38 (Biosource International, Camarillo, CA, USA, code 44–684; 1 : 250; phosphorylated on Thr-180 and Tyr-182). Calcitonin gene-related peptide (CGRP) was detected using a sheep polyclonal antibody at 1 : 2000 (Michael *et al.*, 1997) and mouse anti-S100β was obtained from Sigma. For detection of primary polyclonal antibody, a donkey anti-rabbit FITC (fluorescein isothiocyanate)-conjugated secondary antiserum (Jackson Immunoresearch, West Grove, PA, USA; 1 : 200) was used; for detection of monoclonal antibody a sheep anti-mouse TRITC (tetramethyl rhodamine isothiocyanate) conjugate was used (Jackson Immunoresearch). Double labelling of phosphorylated JNK or total p38 together with CGRP was achieved using standard indirect immunofluorescence. After final washes in phosphate-buffered saline, sections were coverslipped in a phosphate-buffered saline/glycerol solution (1 : 3) containing 2.5% 1,4-diazobicyclo(2,2,2)octane (antifading agent; Vector Laboratories, Burlingame, CA, USA). Immunoreactivity was visualized on a Leica epifluorescence microscope using the Y3 and L4 filter block.

Data analysis

The axonal transport data derived from western blots are presented normalized to control as mean ± SEM ($n = 3$). Data from western blots were derived by subtracting the intermediate nerve segment value (relates possible local changes in synthesis of proteins induced by the ligature) from either the proximal or retrograde accumulation value. Values were also adjusted for background levels of signal derived from intact nerve (Delcroix *et al.*, 1998, 1999). Single comparisons between groups were made using Student's *t*-test. The NT-3 study data, where appropriate and allowing for homogeneity of variances according to the Levene test, were subjected to one-way ANOVA (analysis of variance) using the Statistical Package for the Social Sciences (SPSS/PC+; SPSS, Chicago, IL, USA). Where the *F* ratio gave $P < 0.05$, comparisons between individual group means were made using Tukey's HSD (highly significant difference) multiple range test at significance levels of $P = 0.05$.

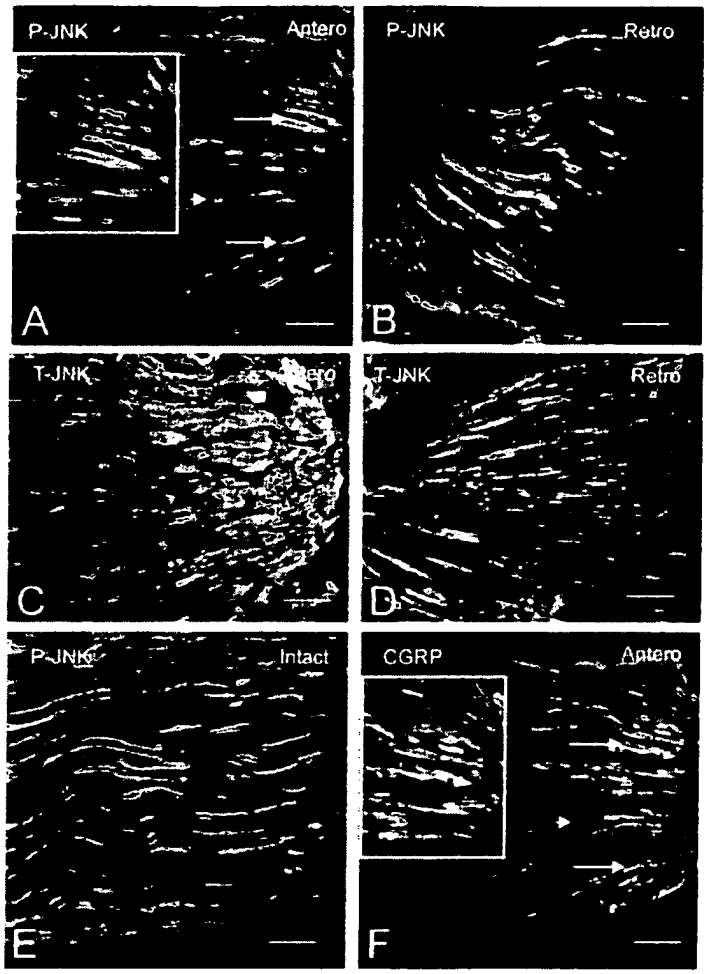

Fig. 1 Immunohistochemical analysis of axonal transport of total and phosphorylated JNK in sciatic nerve. Sciatic nerves of normal rats were subjected to unilateral nerve ligature for 12 h and longitudinal nerve sections stained for phosphorylated or total JNK. As a measure of anterograde (Antero) and retrograde (Retro) axonal transport, the proximal (for anterograde) and distal (for retrograde) nerve segments were stained for (**A**, **B**) phosphorylated JNK (P-JNK) or (**C**, **D**) total JNK (T-JNK). In **E** an intact segment of sciatic nerve has been stained for phosphorylated JNK. In **F** the nerve segment in **A** was double-stained for phosphorylated JNK and CGRP. In **A** and **F** the full arrows indicate areas of colocalization of phosphorylated JNK and CGRP. The arrowhead identifies an area that stains only for phosphorylated JNK. In **A** and **F** areas of key interest have been inserted at ×2 magnification to aid in visualizing areas of colocalization of P-JNK and CGRP. Scale bars are 100 μm.

Results

Immunohistochemical demonstration of axonal transport of JNK and p38 in sciatic nerve

The first aim was to use immunohistochemistry to characterize axonal transport of SAPKs in the sciatic nerve of normal rats. Adult rats underwent unilateral sciatic nerve ligature at mid-thigh for 12 h. Figures 1 and 2 show immunohistochemistry for total and phosphorylated JNK and p38 in ligated and intact sciatic nerve. The detection of phosphorylated JNK and p38 represented the populations of JNK and p38 enzyme that had undergone activation (this also applies for the antibody detection of phosphorylated ERK and ATF2 presented later). Nerve ligation induced bidirectional accumulation of phosphorylated JNK (Fig. 1A and B), total JNK (Fig. 1C and D), phosphorylated p38 (Fig. 2A and B) and total p38 (Fig. 2C and D). Double labelling of the proximal segment (anterograde transport) of nerve showed colocalization of phosphorylated JNK with the neuronal marker CGRP (Fig. 1A and F). The same was true for phosphorylated p38 (Fig. 2A and F). Phosphorylated JNK and p38 were localized to axons but could also be visualized in a limited number of non-neurons, probably Schwann cells (arrowheads in Figs 1A and 2A). Staining for total JNK and p38 was more widespread, with localization to axons but also extensive labelling of non-neuronal elements (Figs 1C, D and 2C, D). Intact sciatic nerve was also stained for phosphorylated JNK (Fig. 1E) and phosphorylated p38 (Fig. 2E), confirming that the staining

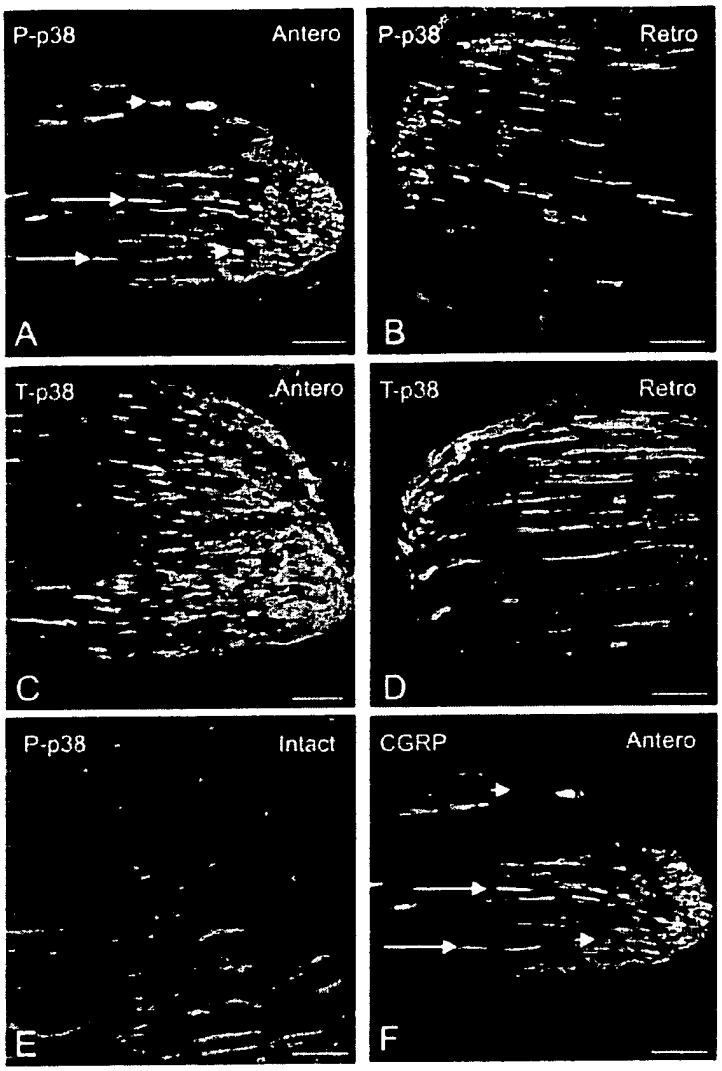

Fig. 2 Analysis of axonal transport of p38 in sciatic nerve using immunohistochemical staining. Longitudinal sections of ligated or intact sciatic nerves from normal rats were stained for phosphorylated or total p38. Anterograde (Antero; proximal) or retrograde (Retro; distal) nerve segments were stained for (**A**, **B**) phosphorylated p38 (P-p38) or (**C**, **D**) total p38 (T-p38). In **E**, intact nerve was stained for phosphorylated p38 and in **F** the nerve segment in **A** was double-labelled for phosphorylated p38 and CGRP. In **A** and **F** the full arrows show areas of costaining and arrowheads indicate sites with no colocalization. Scale bars are 100 μm.

observed in ligated nerve was not simply an artefact generated by the operation. Ligated nerve tissues from STZ-diabetic rats were also analysed using immunohistochemistry and the pattern of staining for JNK and p38 was comparable to that seen in control tissues.

JNK and p38 accumulate at a nerve crush with kinetics indicative of fast axonal transport

The identity and quantity of JNK and p38 (total and phosphorylated enzyme) immunoreactivity was examined by western blotting. At the ligature sites, Fig. 3 shows the westerns blots for the SAPK proteins. The antibodies to total and phosphorylated JNK detected protein species of 56, 54 and 46 kDa, and this most likely reflects the expression of all three of the JNK genes (Gupta *et al.*, 1996). The anti-p38 antibodies detected a single species at ~38–40 kDa. Western blots were quantified and anterograde and retrograde accumulation was calculated by subtracting the intermediate nerve segment values from the values in segments proximal or distal to the ligatures. The rate of retrograde accumulation of phosphorylated JNK and p38 was ~1.0 μm/s and was linear for up to 12 h, which is consistent with accumulation by fast axonal transport (Table 1).

Effect of STZ diabetes on levels of axonal transport of JNK and p38 in sciatic nerve

Age-matched control rats and 8-week STZ-diabetic rats underwent unilateral double crushes of the sciatic nerve of 6 h duration in order to quantify the fast axonal transport levels of the SAPKs [and the downstream target ATF2 (transcription factor); see later]. Nerve segments were homogenized and subjected to western blotting; the levels of accumulation of the SAPKs were determined and the amounts of fast axonal transport of these proteins calculated using the double-ligature paradigm described previously. Figures 3 and 4 show that STZ diabetes induced a significant 2.5- to 3-fold increase in the retrograde axonal transport of the phosphorylated forms of JNK (46 and 54–56 kDa isoforms) and p38 compared with age-matched control rats (all data are in arbitrary units and expressed as fold increase over control: JNK 54–56 kDa isoforms, control 1.0 ± 0.19 versus diabetic 2.5 ± 0.26; p38, control 1.0 ± 0.09 versus diabetic 2.9 ± 0.52; both $P < 0.05$). Retrograde axonal transport of phosphorylated ERK was not similarly affected (see western blot in Fig. 3). The levels of total JNK undergoing retrograde axonal transport were also raised ~1.5-fold compared with controls. For all the SAPKs the levels of anterograde axonal transport were not affected by STZ diabetes (Table 2).

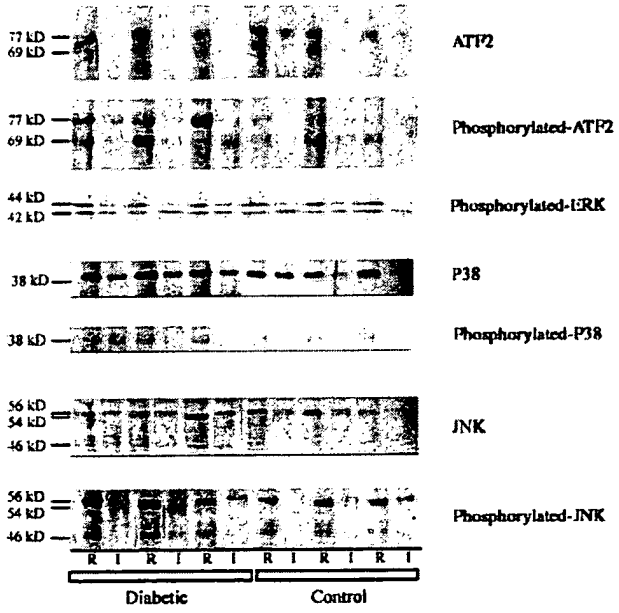

Fig. 3 Western blots showing the effect of STZ diabetes on retrograde accumulation of SAPKs and ATF2 at the site of a sciatic nerve crush. STZ-diabetic and age-matched control rats were maintained for 8 weeks and then subjected to unilateral double sciatic nerve ligature for 6 h. The levels of anterograde and retrograde accumulation of SAPKs and ATF2 were quantified using western blotting. The retrograde (R) and intermediate (I) nerve segments are shown. For the quantification of axonal transport of SAPKs and ATF2, the intermediate nerve segment value (a measure of local synthesis) was subtracted from the retrograde value (local synthesis plus the accumulated protein). The final value was also adjusted for background levels of expression derived from the intact nerve. The anterograde nerve samples did not show any effect of diabetes on axonal transport of the SAPKs or ATF2 (Table 2).

Diabetes induces elevated axonal transport of a SAPK-targeted transcription factor

The effect of diabetes on the axonal transport of the transcription factor ATF2 was also analysed. Both JNK and

Table 1 *JNK and p38 accumulate at a sciatic nerve ligature in a linear fashion with kinetics indicative of fast axonal transport*

Protein kinase	6 h	12 h	12 h : 6 h ratio
P38	41.9 ± 2.35	81.7 ± 6.12	1.95 ± 0.06
JNK p56	29.2 ± 1.74	52.7 ± 1.72	1.82 ± 0.1
JNK p54	26.2 ± 0.7	48.9 ± 2.6	1.88 ± 0.13
JNK p46	24.0 ± 1.22	45.9 ± 4.8	1.90 ± 0.1

Adult rats were subjected to unilateral sciatic ligatures (double) for 6 h or 12 h and accumulation of the phosphorylated forms of JNK (p56, p54 and p46) and p38 were quantified using western blotting. Values are arbitrary units and have been adjusted for local synthesis and background levels. In the case of linear accumulation of protein due to fast axonal transport the ratio of 12 h to 6 h should approach 2.0. Values are mean ± SEM ($n = 3$).

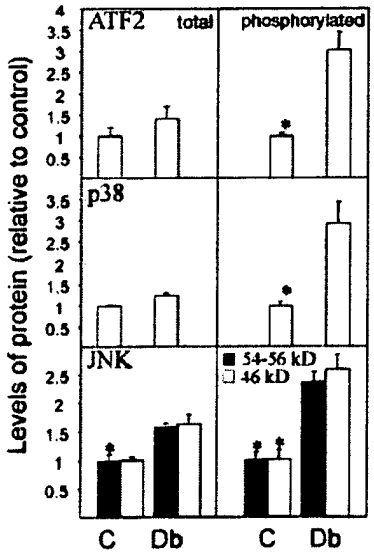

Fig. 4 STZ diabetes elevates retrograde axonal transport of phosphorylated JNK, p38 and ATF-2. The data were derived from the western blots presented in Fig. 3. Control (C) and STZ-diabetic (Db) rats were maintained for 8 weeks and axonal transport was measured using a unilateral double ligature of the sciatic nerve for 6 h. Western blots of nerve segments were performed for phosphorylated and total JNK (46 kDa; open columns) and 54–56 kDa (solid columns) isoforms of JNK. Axonal transport of p38 and ATF-2 was calculated as described in Methods. Values are expressed as mean and SEM ($n = 3$) and all data have been normalized to the respective control value. *$P < 0.05$ for control versus STZ-diabetic (t-test).

p38, when activated, induce the phosphorylation of ATF2 and enhance transcriptional activity (Whitmarsh and Davis, 1996; Davis, 2000; Harper and LoGrasso, 2001; Weston and Davis, 2002). Figures 3 and 4 show that diabetes induced a significant 3-fold elevation in the retrograde axonal transport of phosphorylated ATF2 (control 1.0 ± 0.07 versus diabetic 3.0 ± 0.41; $P < 0.05$). Levels of retrograde transport of total ATF2 were unaffected, as was anterograde transport of phosphorylated ATF2 (data not shown).

Table 2 *Anterograde accumulation of phosphorylated ERK, JNK and p38 at a sciatic nerve ligature is not affected by diabetes*

Protein kinase	Control	STZ-diabetic
P-ERK	1.0 ± 0.05	1.0 ± 0.02
P-JNK	0.85 ± 0.11	1.0 ± 0.11
P-p38	1.01 ± 0.1	1.0 ± 0.07

Adult rats were subjected to unilateral sciatic nerve ligatures (double) for 6 h and anterograde accumulation of the phosphorylated forms of ERK, JNK (p56 and p54 kDa) and p38 were quantified using western blotting. Data have been normalized to control and values are mean ± SEM ($n = 3$). Levels of anterograde axonal transport of total p38 and JNK were similarly unaffected by diabetes.

Localization of SAPKs to axon and/or Schwann cell

We next confirmed the localization of activated JNK and p38 to the axon in normal and diabetic nerve. Figure 5 shows immunohistochemical staining for activated JNK and p38 in transverse sections of sciatic nerve from normal (Fig. 5A and C) and STZ-diabetic (Fig. 5B and D) animals. The staining for activated JNK was highly enriched in the axonal area, with little staining of Schwann cells in normal and diabetic animals (Fig. 5A and B). The staining for activated p38 was more widespread, with significant staining of Schwann cells and weak axonal staining in normal animals (S100 staining in Fig. 5C and F). In STZ-diabetic animals the p38 staining showed a stronger axonal signal (Fig. 5D). The level of

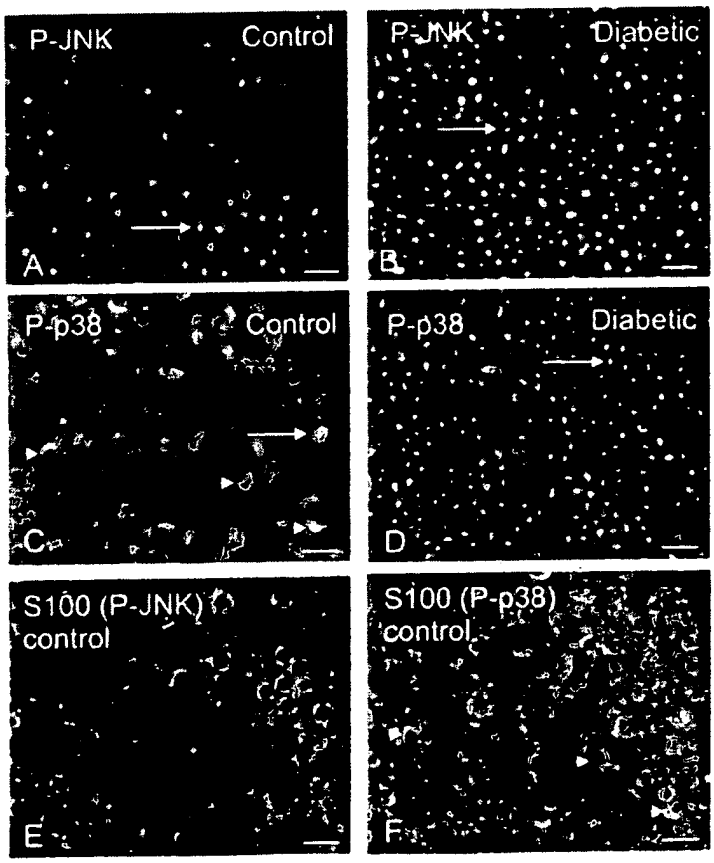

Fig. 5 Localization of phosphorylated JNK and p38 to axons and Schwann cells of sciatic nerve. Transverse sections of sciatic nerve from (**A**, **C**) control and (**B**, **D**) 14-week STZ-diabetic animals, showing immunostaining for (**A**, **B**) phosphorylated JNK and (**C**, **D**) phosphorylated p38. In **E** and **F** the control nerve sections have been double-stained for S100β. Full arrows indicate areas of axonal immunostaining and arrowheads indicate areas of Schwann cell staining. Scale bars are 100 μm.

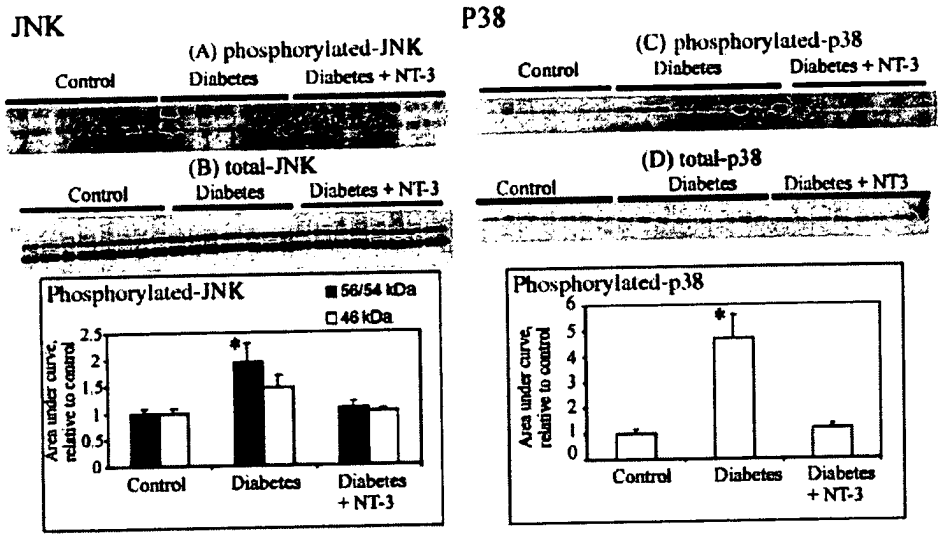

Fig. 6 NT-3 prevents the diabetes-induced activation of JNK and p38 in sciatic nerve. Diabetic animals (14 weeks) were treated with 5 mg/kg NT-3 for the final 10 weeks of the study and then sciatic nerve segments were subjected to quantitative western blotting. Western blots and data are shown for (**A**) phosphorylated JNK (54–56 and 46 kDa isoforms), (**B**) total JNK (all isoforms), (**C**) phosphorylated p38 and (**D**) total p38. Values are presented relative to age-matched control animals and are mean and SEM (n = 7–9). *$P < 0.05$ for diabetic versus other groups (one-way analysis of variance).

staining for both activated JNK and p38 was clearly higher in the sciatic nerve of STZ-diabetic animals.

When analysing whole-nerve expression of SAPKs, we believe it is correct to assume that JNK expression has a mainly axonal origin, whereas the p38 signal has contributions from axon and Schwann cell. This fact should be understood when interpreting the studies presented below on NT-3 effects on SAPK activation in diabetes.

NT-3 prevents the diabetes-induced elevation of JNK and p38 activation in sciatic nerve

In order to determine if the activation of SAPKs in diabetes was related to loss of neurotrophic support, we treated 14-week STZ-diabetic animals with 5 mg/kg human recombinant NT-3 for the final 10 weeks of the study. Sciatic nerve homogenates were subjected to quantitative western blotting and probed for the SAPKs (Fig. 6). Phosphorylation of JNK and p38 was significantly elevated 1.95- and 4.7-fold, respectively, in sciatic nerve of STZ-diabetic animals. The 56/54 and 46 kDa isoforms of JNK were elevated, the 56/54 kDa isoforms being most clearly raised (Fig. 6A). Treatment with NT-3 significantly prevented this diabetes-induced activation of JNK and p38 (Fig. 6) (phosphorylated JNK 46 kDa isoform, control 1.0 ± 0.09, diabetic 1.95 ± 0.35, diabetic + NT-3 1.09 ± 0.12; $P < 0.05$ diabetic versus others; phosphorylated p38, control 1.0 ± 0.16, diabetic 4.7 ± 0.9, diabetic + NT-3 1.19 ± 0.18; $P < 0.05$ diabetic versus others). STZ diabetes also increased the levels of total JNK (54–56 kDa isoform; Fig. 6B), although this was not statistically significant (control 1.0 ± 0.21, diabetic 1.69 ± 0.26, diabetic + NT-3 1.41 ± 0.34). Total levels of JNK (46 kDa isoform) and p38 were not affected by STZ diabetes (Fig. 6B and D) (data not shown).

Discussion

The results demonstrate for the first time that SAPKs undergo fast axonal transport in peripheral nerve and that the activation status of these enzymes can be elevated in an animal model of sensory neuropathy, the STZ-diabetic rat. A critical observation is that the diabetes-induced enhancement of JNK and p38 activation is restricted to the retrograde component of axonal transport; this gives directionality to the stress signal. Furthermore, activation of JNK and p38 was specific, with no effect on ERK. These results are novel because they highlight one mechanism whereby stress signals that are generated peripherally can be translocated to the sensory neuron soma. Interestingly, as part of the stress response we demonstrate that a target transcription factor of JNK and p38, namely ATF2, is activated peripherally and transported axonally to the neuronal cell body. Treatment of STZ-diabetic rats with NT-3 prevented activation of JNK and p38 in sciatic nerve, implying a role for neurotrophin withdrawal in the mechanism of activation of the SAPKs.

SAPKs and neurodegeneration

Axotomy of adult sensory neurons results in a perikaryal response highlighted by the phosphorylation of JNK and related elevated activation and expression of c-jun; however, a significant level of cell death does not occur (Jenkins *et al.*,

1993; Gold *et al.*, 1994; Kenney and Kocsis, 1998). The loss of neurotrophic support following axotomy may be instrumental in this JNK activation. In rat models of sensory neuropathy there is activation of JNK, p38 and ERK in DRG and sural nerve (Fernyhough *et al.*, 1999). This could be derived from diminished neurotrophic support (Fernyhough *et al.*, 1995, 1998). In cultured adult sensory neurons, high concentrations (>25 mM) of glucose triggered activation of JNK. This suggests that, in STZ-diabetic or Bio-Breeding diabetic rats, a critical factor controlling the neuronal stress response is also hyperglycaemia (Tomlinson, 1999; Purves *et al.*, 2001).

Hyperglycaemia, neurotrophic support and activation of SAPKs

In kidney, in response to high concentrations of glucose the GTPase Cdc42 is activated via an adapter protein, Gene 33, and mediates sustained activation of JNK (Makkinje *et al.*, 2000). The mobilization of GTP-Cdc42 by high glucose is JNK-dependent, i.e. JNK triggers its own long-term activation. In neurons there is evidence that high glucose can induce oxidative stress and may be a central mediator of SAPK activation (Tomlinson, 1999; Purves *et al.*, 2001). High glucose concentrations result in elevated oxidative phosphorylation and the subsequent production of reactive oxygen species may induce diabetic complications (Nishikawa *et al.*, 2000). The reactive oxygen species-dependent activation of SAPKs may be one critical pathway leading to neurodegeneration (Tomlinson, 1999; Purves *et al.*, 2001). Furthermore, in neurons the loss of trophic support may result in lack of ligand binding to trk receptors and $p75^{NTR}$. This can induce activation of the death domain of $p75^{NTR}$ and activation of JNK (Kaplan and Miller, 2000; Harrington *et al.*, 2002). Additionally, neurotrophins activate protein kinase B/Akt, which is a negative modulator of the scaffold protein JNK inhibitory protein 1 (JIP-1), and lack of neurotrophin action will therefore lead to downregulation of Akt and consequently the enhancement of JNK activation (Kim *et al.*, 2002).

We have described a retrograde stress signal that reflects a neurodegenerative process that is operating along the length of the axon and/or is related to a nerve ending/synapse interaction with target. Hyperglycaemia will occur along the whole length of the nerve and therefore activation of SAPKs in the axon will occur at any point. The activated SAPKs are loaded specifically onto the retrograde transport machinery (to be discussed later). Impairment in trophic support could also occur along the whole length of the axon. However, it is most likely that a target tissue-related loss of neurotrophic support is involved (Fernyhough and Tomlinson, 1999). NGF and NT-3 expression is downregulated in muscle and skin in STZ-diabetic rats and, therefore, reduced occupancy of trk receptors and $p75^{NTR}$ at nerve endings may contribute to a distally generated stress signal.

NT-3-dependent normalization of SAPK activation

The results show that NT-3 prevented the diabetes-induced elevation in JNK and p38 activation in intact nerve. The complete reversal of activation of JNK and p38 in nerve by NT-3 was surprising. In cultured embryonic cortical neurons, brain-derived neurotrophic factor and NT-3 treatment were shown to elevate neurofilament H phosphorylation, possibly via increased activation of JNK and ERK (Tokuoka *et al.*, 2000). Furthermore, only ~20% of sensory neurons in adult DRG express trkC receptors. The nociceptive/mechanoreceptive population, which constitutes ~80% of the neuronal DRG cells, do not express trkC receptors (Michael *et al.*, 1999). However, at 5.0 mg/kg, NT-3 may be affecting SAPK activation in a wide range of sensory neuron phenotypes via binding to trkC, trkA and/or $p75^{NTR}$ (Belliveau *et al.*, 1997; Dechant *et al.*, 1997). An alternatively spliced form of trkA has high affinity for NT-3; however, its expression in sensory neurons is unknown (Clary and Reichardt, 1994). The ability of NT-3 to interact with trkA is also partly dependent on $p75^{NTR}$. Extracellular truncation or loss of expression of $p75^{NTR}$ permits NT-3 binding to trkA (Mischel *et al.*, 2001), and this will be encouraged in sensory neurons of diabetic animals, in which $p75^{NTR}$ levels of expression are reduced (Delcroix *et al.*, 1998).

Mechanism of axonal transport of SAPKs

In fibroblasts, JNK is localized to microtubules and in close proximity to kinesin (Nagata *et al.*, 1998). The scaffold protein JIP-1, an important regulator of JNK function in neurons and non-neurons, is present in axons and growth cones of neurons and undergoes axonal transport in non-neurons (Verhey *et al.*, 2001; Whitmarsh *et al.*, 2001). JIP-1, -2 and -3 bind to the COOH-terminus of the kinesin light chain and regulate JNK function via binding. Therefore, kinesin-dependent transport of a JIP/JNK complex is feasible (Verhey *et al.*, 2001). This would account for the anterograde component; however, no work has been done linking dynein with axonal transport of JIP. Howe and colleagues have recently shown that NGF signalling via endosomes containing aggregates of signalling complexes of the Ras-mitogen-activated protein kinase pathway (Howe *et al.*, 2001). There is no direct evidence that JNK or p38 is retrogradely transported in this manner; however, trkA can bind to cytoplasmic dynein (Yano *et al.*, 2001) and one study demonstrates that JIP-2 can bind to a transmembrane receptor (the reelin receptor ApoER2) (Stockinger *et al.*, 2000). Furthermore, dynein light chain can bind to the neuronal scaffold protein guanylate kinase-associated protein (GKAP) (Fan *et al.*, 2001), which, in turn, can associate with membrane-associated guanylate kinases (MAGUK), which are proteins that have an Src homology 3 domain and may act as a proximal focus of signalling pathways downstream from trkA and upstream from JNK activation (Shin *et al.*, 2000). It

can be proposed that retrograde transport of SAPKs is mediated via trk receptor association. Therefore, the diabetes-induced modulation of activation of SAPKs is due to modification of trophic factor activation of trk receptors and alteration of the activation state of the associated signalling complexes.

Downstream consequences of SAPK activation in diabetic neuropathy

Activation of SAPKs, especially JNK and p38, is associated with neurodegeneration in PC12 cells and sympathetic neurons (Xia *et al.*, 1995; Deshmukh and Johnson, 1997). Axotomy of sensory neurons activates JNK, but cell death is not the result; in fact, neurons survive and undergo axonal regeneration (Kenney and Kocsis, 1998). Exposure of cultured adult sensory neurons to high glucose concentrations results in JNK activation but cell death does not occur (Purves *et al.*, 2001). In non-neurons the inhibitor of apoptosis family of proteins protect cells from TNF-α-induced cell death via activation of JNK1 (Sanna *et al.*, 2002). Therefore, activation of JNK may be a protective pathway against cell death during specific stages of development. The situation with activation of p38 is more straightforward. Exposure of cultured sensory neurons to oxidative stress results in cell death and activation of p38; inhibition of the latter affords protection (Purves *et al.*, 2001). Interestingly, STZ-diabetic rats exhibit allodynia and hyperalgesia, and Ji *et al.* (2002) have shown that p38 is activated in small-fibre sensory neurons in response to inflammation (Calcutt, 2002; Ji *et al.*, 2002). During inflammatory pain or heat hypersensitivity, the activation of p38 involves enhanced retrograde transport of NGF; therefore, activation of p38 in STZ diabetes is via an alternative mechanism since NGF-dependent trophic support is down-regulated in diabetes. Therefore, inhibition of p38 protects not only against oxidative stress-induced neurodegeneration but also against hyperalgesia, and so blockage of p38 activation should be an important target for drug therapy in diabetic neuropathy.

Conclusions

In summary, we demonstrate for the first time that JNK and p38 are axonally transported in neurons. This transport process is modulated by stressful conditions, such as those found in type I diabetes, and may mediate the transfer of stress signals from periphery to the sensory neuron cell body. The diabetes-induced stress signal may involve a loss of trophic support. Finally, diabetes induces coordinate induction of JNK and p38 in axons, and the level of neurodegeneration may depend on the relative activities of these two signalling pathways.

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